Faculty of Resource Science and Technology

ISOLATION AND EXPRESSION OF HYDROXYPHENYLPYRUVATE REDUCTASE (*HPPR*) IN *ORTHOSIPHON ARISTATUS*

Zuliza Binti Ahmad

Master of Science

(Plant Biotechnology)

2014

Isolation and expression of hydroxyphenylpyruvate reductase (*HPPR*) in *Orthosiphon aristatus*

ZULIZA BINTI AHMAD

A thesis submitted

in fulfillment of the requirement for the degree of

Master of Science

Faculty of Resources Science and Technology

UNIVERSITI MALAYSIA SARAWAK

2014

ACKNOWLEDGEMENTS

First and foremost I wish to extend my sincerest gratitude to my supervisor, Associate Professor Dr. Hairul Azman Roslan, who has supported me with his patience, knowledge, advice and guidance throughout my thesis. It was a great opportunity to work with an experienced scientist like him.

I would also like to acknowledge Nabella Holling, Jerry Gerunsin, Liyana Ismail, Lee Jong Jen, Norzainizul Julaihi and Wee Ching Ching for the companionship and for their willingness to share their bright ideas, which has been valuable for my research. I had a really pleasant time working together.

Furthermore I would like to thank all the laboratory assistants especially Mr Azis for helping with preparation of materials for the experiments.

Deepest thanks and appreciation to my parents, Ahmad Suhaili and Rokiah Ariffin for all the support and encouragement in pursuing this master degree. Thank you to all family members and friends especially my husband, Ahmad Alinafia Alias and my son Aqil Zafran for supporting my work and for the motivation throughout my study.

Last but not least I would like to acknowledge UNIMAS for the Zamalah Scholarship and Ministry of Science, Technology and Innovation (MOSTI) for the tuition fee scholarship.

ABSTRACT

Orthosiphon aristatus or locally known as '*Misai Kucing*' is a medicinal herb that contains rosmarinic acid. Rosmarinic acid is a natural plant constituent that can be found in *Lamiaceae* herbs and has been proven to have antioxidant, antiviral and antibacterial properties. In the biosynthetic pathway, hydroxyphenylpyruvate reductase (*HPPR*) is one of the enzymes in production of rosmarinic acid. It is responsible in reducing 4-hydroxyphenylpyruvate to 4-hydroxyphenyllactate in dependence of NAD(P)H. In this study, partial length of the *HPPR* gene cDNA was isolated. Reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE) technique were used in the experiment. Primers were designed based on *HPPR* gene from other species obtained from the NCBI database. The internal fragment nucleotide was obtained and the size of the sequence is 583 bp. The 3' and 5' end of the *HPPR* gene was successfully isolated. Sequencing result shows that the 3' and 5' end band contains 842 bp and 791 bp nucleotides respectively. BLAST search revealed that the 3' and 5' end band nucleotide sequence of *O. aristatus HPPR* had sequence similarities to *HPPR* genes from other plant species (86 to 89% homology). *In silico* analysis was performed where the 3' end and 5' end fragments were merged. The open reading frame (ORF) contains 924 bp which is equivalent to a putative amino acid of 307 long. In this study, it is shown that the deduced protein sequence contains a D-isomer specific 2-hydroxyacid dehydrogenases NAD-binding signature and a putative catalytic domain. These domains are typical domains for the family of D-isomer-specific 2-hydroxyacid dehydrogenases. UV light is an abiotic stimulus that has been shown to induce accumulation of rosmarinic acid in plants. In the UV analysis study, it is shown that

UV treatment on *O. aristatus* plant increase expression of *HPPR*. The amplification product intensity is lowest in control plant and is highest in plants exposed to UV for 60 minutes.

ABSTRAK

Orthosiphon aristatus atau nama tempatannya Misai Kucing merupakan herba perubatan yang mengandungi asid rosmarinik. Asid rosmarinik adalah kandungan semulajadi tumbuhan yang boleh didapati dalam herba jenis *Lamiaceae* yang terbukti mengandungi ciri-ciri antioksidan, antiviral dan antibakteria. Dalam proses biosintesisnya, hydroxyphenylpyruvate reductase (*HPPR*) merupakan salah satu enzim dalam penghasilan asid rosmarinik. Ia berfungsi dalam menurunkan 4-hydroxyphenylpyruvate kepada 4-hydroxyphenyllactate dengan adanya NAD(P)H. Dalam kajian ini, sebahagian jujukan cDNA bagi gen *HPPR* telah dipencilkan. Kaedah 'Reverse transcription-polymerase chain reaction (RT-PCR)' dan 'Rapid amplification of cDNA ends (RACE)' telah digunakan untuk eksperimen. 'Primer' dihasilkan berdasarkan gen *HPPR* daripada spesies lain yang diperolehi di database NCBI. Fragmen nukleotida dalaman telah diperolehi dan saiz jujukannya ialah 583 bp. Hujung 3' dan 5' gen *HPPR* telah berjaya dipencilkan dan hasil penjujukan menunjukkan fragmen mengandungi 842 bp dan 791 bp nukleotida. Pencarian 'BLAST' menunjukkan bahawa jujukan nukleotida *HPPR O. aristatus* mempunyai persamaan dengan gen *HPPR* daripada spesies tumbuhan yang lain (persamaan 86 ke 89%). Analisis *'in silico'* telah dilakukan di mana fragmen hujung 3' dan 5' telah digabungkan. 'Open reading frame' (ORF) tersebut mengandungi 942 bp yang bersamaan dengan asid amino jangkaan yang sepanjang 307. Kajian ini menunjukkan jangkaan jujukan protein tersebut mengandungi tanda perlekatan D-isomer specific 2-hydroxyacid dehydrogenases NAD dan domain catalytic. Domain ini adalah domain yang biasa didapati dalam famili D-isomer-specific 2-hydroxyacid dehydrogenases. Cahaya UV merupakan stimulus abiotik yang telah dibuktikan

dapat meningkatkan penghasilan asid rosmarinik pada tumbuhan. Dalam analisis UV, ia telah menunjukkan bahawa rawatan UV ke atas tumbuhan *O. aristatus* meningkatkan ekspresi *HPPR*. Intensiti produk yang diamplifikasi dilihat paling rendah pada tumbuhan kawalan dan paling tinggi pada tumbuhan yang didedahkan dengan cahaya UV selama 60 minit.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

2-4-D	2,4-dichlorophenoxyacetic acid
4CL	hydroxycinnamic acid:coenzyme A ligase
ACP	annealing control primer
BA	6-benzyl adenine
BLAST	Basic Local Alignment Search Tool
bp	base pair
$CaCl_2$	calcium chloride
CAH	cinnamic acid 4-hydroxylase
cDNA	complementary DNA
cds	coding DNA sequence
Chl:Iaa	chloroform:isoamylalcohol
CTAB	cetyl trimethylammonium bromide
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EB	extraction buffer
EDTA	ethylenediaminetetraacetic acid
ef-1α	elongation factor 1 alpha
g	gram
g/kg	gram per kilogram
g/L	gram per liter
GST	glutathione-S-transferase
HCl	hydrochloric acid

HDL	high-density lipoprotein
HPPD	hydroxyphenylpyruvate dioxigenase
HPPR	hydroxyphenylpyruvate reductase
IAA	indole-acetic acid
IBA	indole-3-butyric acid
IPTG	isopropyl-ß-D-thiogalactopyranoside
L	liter
LB	Luria-Bertani
LiCl	lithium chloride
µL	microliter
µg/mL	microgram per microliter
µM	micromolar
M	molar
mg/L	milligram per liter
$MgCl_2$	magnesium chloride
mL	mililiter
mM	milimolar
MOSTI	Ministry of Science, Technology and Innovation
mRNA	messenger RNA
MS	Murashige and Skoog
MW	molecular weight
NAA	1-napthalene acetic acid
NaAc	sodium acetate
NaCl	sodium chloride
NAD(P)H	reduced form of nicotinamide adenine dinucleotide phosphate

CHAPTER ONE

INTRODUCTION

1.1 Background

Herbal products are getting more popular as alternative medicines and food supplements. Herbal medicine is defined as preparations derived from plants and fungi in the form of alcoholic extraction or decoction which is used in prevention or treatment of diseases (Linde *et al*., 2001). According to the estimation by World Health Organization (WHO), 70-80% of the world population uses herbal products in medication (Eichhorn *et al*., 2011). The total global herbal drug market is estimated to be US$62 billion in 2010 (Citarasu, 2010) and the trade is expected to increase to US$5 trillion by 2050 (Booker *et al*., 2012). Despite the availability of synthetic chemical, plant source for pharmaceutical products is still preferred because it is natural, safe, readily available, and cost less (Wasim *et al*., 2011).

The therapeutic effects of herbal medicines are mainly attributed to their bioactive secondary metabolites such as polyphenols, alkaloids, saponin and terpenes. Several secondary metabolites derived from plants have been proven to be important in the pharmaceutics for example vinblastine and vincristine which is derived from *Catharanthus roseus*. Each plant species has a unique set of secondary metabolites. The structure of the secondary metabolite is complex and their chemical synthesis is

not economically feasible (Oksman-Caldentey & Inzé, 2004). The biosynthetic pathways of these secondary metabolites are long and mediated by various enzymes. This leads to the need for molecular study on the enzyme that is responsible for production of the plants' bioactive chemical constituent.

Orthosiphon aristatus is a medicinal herb that has been widely commercialized in production of food supplements due to its bioactive content. Demand for herbal medicinal products is increasing due to general public awareness and interest in health care. In 1999, the sale of herbal products in Malaysia was estimated at RM 4.6 billion (Jamia, 2006). In Malaysia, this plant has been cultivated for local commercial products in the form of tea sachets, drinks, raw herbs, tablets and capsules (Abdullah *et al*., 2012). These herbal products is claimed to have health benefits such as detoxification, weight loss, for treatment of hypertension, gout, kidney stone, and inflammation (Awale *et al*., 2002).

Extensive scientific studies are currently ongoing for this highly potential plant. Several studies have been reported on the plant commencing with phytochemical screening (Tezuka *et al.,* 2000; Awale *et al*., 2002; Hossain & Ismail, 2011) followed by pharmacological (Olah *et al*., 2003), genotoxicity (Chin *et al*., 2008; Muhammad *et al*., 2011) and propagation studies (Elangomathavan *et al*., 2003; Lee & Chan, 2004; Ling *et al*., 2009). Studies reveal that this plant has various useful biological activities such as antioxidant (Khamsah *et al*., 2006; Abdelwahab *et al*., 2011),

antimicrobial (Tong *et al*., 2011), antibacterial (Ho *et al*., 2010), anti-fungal (Hossain *et al*., 2008), anti-pyretic (Yam *et al*., 2009), anti-obesity (Son *et al*., 2011), chemopreventive (Salleh *et al*., 2011), hepatoprotective (Maheswari *et al*., 2008) and diuretic properties (Arafat *et al*., 2008; Adam *et al*., 2009), thus providing scientific support to its use in traditional medicine. Most of the papers dealing with bioactive properties refer these effects to the major compound in *O. aristatus* which includes rosmarinic acid (Arafat *et al*., 2008; Ho *et al*., 2010).

At present, the specific biosynthetic pathway for the production of rosmarinic acid in *O. aristatus* is still not clear. Detailed knowledge needs to be obtained to understand the regulatory role of the enzymes in its biosynthetic pathway. The hydroxyphenylpyruvate reductase (*HPPR*) is the first specific enzyme in the biosynthesis of rosmarinic acid (Kim *et al*., 2004). Basic information regarding the molecular aspects of the *HPPR* gene in *O. aristatus* will be useful in determining the presence of the enzyme and verifying its proposed role in the plant. Furthermore this molecular information can be useful for future biotechnological application in *O. aristatus*. In order to study the regulation of RA biosynthesis in *O. aristatus*, isolation and expression of *HPPR* was characterized biochemically.

1.1.1 Research Objectives

The objectives of this study are:

i. To isolate the *HPPR* cDNA involved in the biosynthesis of rosmarinic acid in *O. aristatus*.

ii. To characterise the *HPPR* cDNA isolated from *O. aristatus*.

iii. To examine the effect of UV exposure on *O. aristatus HPPR* mRNA via reverse transcription polymerase chain reaction (RT-PCR).

1.2 Literature review

1.2.1 *Orthosiphon aristatus*

Orthosiphon aristatus (Bl.) Miq. [syn.: *O. grandiflorus* Bold., *O. spicatus* (Thumb) Bak, *O. spiralis*, *O. stamineus* Benth.] is commonly known as Misai Kucing (Malaysia), Kumis Kucing or Remujung (Indonesia), Kumis Ucing (Sudanese), Java tea (English), Thé de Java (France), Yaa Nuat Maeoo (Thailand), Balbas-pusa or Kabling-gubat (Philippines), Hnwàd Méew (Laos), R[aa]u M[ef]o (Vietnam), Neko no hige (Japan) and Kapan Prey in Cambodia (Akanae *et al*., 2010). It belongs to the family Lamiaceae or Labiatea (Chan & Loo, 2006). This plant is native to tropical Asia. It can easily be cultivated through stem cuttings, seeds and thrives in well-drained soils and full sunlight (Elangomathan *et al*., 2003). It is usually planted in home gardens for medicinal or ornamental purposes. It can also be found in the wild, growing at forest edges and along the roadsides.

This perennial herb grows to about 0.4 to 1.5 m high (Abdelwahab *et al*., 2011). The leaves are dark green, simple, with a lanceolate leaf blade and a serrate margin (Chan & Loo, 2006). They are arranged in opposite pairs with relatively short petiole which are reddish purple in colour (Adam *et al*., 2009). The stem is quadrangle, erect, branches profusely and is reddish in colour. The inflorescence is borne on verticils of about 16 cm in length. Flowers are hermaphrodite with irregular flower symmetry. Flowers have long protruding stamens, making it look like cat's whiskers. There are two calyx lobes, which are greenish red in colour and measuring about 6 mm in length. There are two corolla lobes which are light violet in colour and covered with minute hairs (Jaganath & Ng, 2000). There are two varieties based on floral colour which is the white variety and the purple variety (Chan & Loo, 2006). Figure 1.1 shows the *O. aristatus* plant. The scientific classification for the plant is shown in Table 1.1.

Figure 1.1: *Orthosiphon aristatus.*

Table 1.1: Scientific classification for *O. aristatus.*

Kingdom:	Plantae
Subdivision:	Angiosperms
Class:	Eudicots
Subclass:	Asterids
Order:	Lamiales
Family:	Lamiaceae
Genus:	*Orthosiphon*
Species:	*O. aristatus*

Extensive works have been done in analysing the plant compounds. More than fifty compounds have been isolated and identified in *O. aristatus*, which includes 7,3',4'-tri-*O*-methylluteolin, eupatorin, sinensetin, 5-hydroxy-6,7,3',4'-tetramethoxyflavone, salvigenin, ladanein, tetramethylscutellarein, 6-hydroxy-5,7,4'-trimethoxyflavone, vomifoliol, aurantiamide acetate, rosmarinic acid, caffeic acid, oleanolic acid, ursolic acid, betulinic acid, β-sitosterol, orthosiphols, staminols, staminolactones, and norstaminol A (Tezuka *et al*., 2000; Hossain & Ismail, 2011). Novel compounds unique to the plant cultivation location has also been isolated for example norstaminane- and isopimarane-type diterpenes from Okinawa (Awale *et al*., 2002), triterpenes and prenylated flavonoids from Malaysia (Hossain & Ismail, 2011). The most abundant polyphenol present in the aqueous methanol extract of *O. aristatus* leaves has been determined to be rosmarinic acid (Sumaryono *et al*., 1991; Akowuah *et al*., 2005; Chin *et al*., 2009).

In Southeast Asia, *O. aristatus* has been used for centuries in traditional medicine as diuretic, and for treatment of fever, epilepsy, gallstones, hepatitis, rheumatism, hypertension, syphilis, gonorrhea, tonsilitis, hepatitis, gout and diabetes (Akowuah *et al*., 2005; Kiong *et al.*, 2008). Fresh or dried leaves are infused in warm water and this tea is taken as healthy beverage (Abdullah *et al*., 2012). In countries such as Malaysia, Indonesia, Thailand and Vietnam the tea is commonly used in treatment of kidney diseases because of its diuretic properties (Arafat *et al*., 2008)

Various ranges of scientific studies support the traditional use of the plant. A study by Yam *et al.* (2009) has confirmed that the methanol extract of *O. aristatus* possess a significant anti-pyretic activity in rats. Several studies indicate that the plant exhibited significant radical scavenging or antioxidant (Akowuah *et al.*; 2005, Khamsah *et al*., 2006), antiapoptotic (Abdelwahab *et al*., 2011), antimicrobial (Tong *et al*., 2011) and antibacterial activity (Ho *et al*., 2010). The plants' extract has also been shown to display great potential in anti-fungal activity (Hossain *et al*., 2008) The plant has also been proven to have chemopreventive activity and has a potential to be developed as an agent for human liver cancer prevention (Salleh *et al*., 2011).

Apart from that, *O. aristatus* extract can also reduce appetite which can be applicable for body weight control (Son *et al*., 2011). A study by Maheswari *et al*. (2008) showed that *O. aristatus* has hepatoprotective activity on liver damage caused by paracetamol in rats. Methanol extracts of *O. aristatus* has also been reported to